PATENT COOPERATION TREATY

From the INTERNATIONAL SEARCHING AUTHORITY

PCT

NOTIFICATION OF TRANSMITTAL OF THE INTERNATIONAL SEARCH REPORT OR THE DECLARATION

(PCT Rule 44.1)

To:
CHEONG & MIRANDAH
Attn. MIRANDAH, Gladys
111 North Bridge Road
22-01/02/03 Peninsula Plaza
Singapore 179098
SINGAPORE

Date of mailing *(day/month/year)* 14/03/2001	
Applicant's or agent's file reference GM/MC/R33-92	**FOR FURTHER ACTION** See paragraphs 1 and 4 below
International application No. PCT/SG 00/00111	International filing date *(day/month/year)* 01/08/2000

Applicant

INSTITUTE OF MOLECULAR AGROBIOLOGY et al.

1. [X] The applicant is hereby notified that the International Search Report has been established and is transmitted herewith..

Filing of amendments and statement under Article 19:
The applicant is entitled, if he so wishes, to amend the claims of the International Application (see Rule 46):

When? The time limit for filing such amendments is normally 2 months from the date of transmittal of the International Search Report; however, for more details, see the notes on the accompanying sheet.

Where? Directly to the International Bureau of WIPO
34, chemin des Colombettes
1211 Geneva 20, Switzerland
Fascimile No.: (41-22) 740.14.35

For more detailed instructions, see the notes on the accompanying sheet.

2. [ ] The applicant is hereby notified that no International Search Report will be established and that the declaration under Article 17(2)(a) to that effect is transmitted herewith.

3. [ ] **With regard to the protest** against payment of (an) additional fee(s) under Rule 40.2, the applicant is notified that:

[ ] the protest together with the decision thereon has been transmitted to the International Bureau together with the applicant's request to forward the texts of both the protest and the decision thereon to the designated Offices.

[ ] no decision has been made yet on the protest; the applicant will be notified as soon as a decision is made.

4. **Further action(s):** The applicant is reminded of the following:

Shortly after **18 months** from the priority date, the international application will be published by the International Bureau. If the applicant wishes to avoid or postpone publication, a notice of withdrawal of the international application, or of the priority claim, must reach the International Bureau as provided in Rules 90*bis*.1 and 90*bis*.3, respectively, before the completion of the technical preparations for international publication.

Within **19 months** from the priority date, a demand for international preliminary examination must be filed if the applicant wishes to postpone the entry into the national phase until 30 months from the priority date (in some Offices even later).

Within **20 months** from the priority date, the applicant must perform the prescribed acts for entry into the national phase before all designated Offices which have not been elected in the demand or in a later election within 19 months from the priority date or could not be elected because they are not bound by Chapter II.

Name and mailing address of the International Searching Authority	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL-2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Heike Zoglauer

Form PCT/ISA/220 (July 1998)

NOTES TO FORM PCT/ISA/220

These Notes are intended to give the basic instructions concerning the filing of amendments under article 19. The Notes are based on the requirements of the Patent Cooperation Treaty, the Regulations and the Administrative Instructions under that Treaty. In case of discrepancy between these Notes and those requirements, the latter are applicable. For more detailed information, see also the PCT Applicant's Guide, a publication of WIPO.

In these Notes, "Article", "Rule", and "Section" refer to the provisions of the PCT, the PCT Regulations and the PCT Administrative Instructions respectively.

INSTRUCTIONS CONCERNING AMENDMENTS UNDER ARTICLE 19

The applicant has, after having received the international search report, one opportunity to amend the claims of the international application. It should however be emphasized that, since all parts of the international application (claims, description and drawings) may be amended during the international preliminary examination procedure, there is usually no need to file amendments of the claims under Article 19 except where, e.g. the applicant wants the latter to be published for the purposes of provisional protection or has another reason for amending the claims before international pbulication. Furthermore, it should be emphasized that provisional protection is available in some States only.

What parts of the international application may be amended?

Under Article 19, only the claims may be amended.

During the international phase, the claims may also be amended (or further amended) under Article 34 before the International Preliminary Examining Authority. The description and drawings may only be amended under Article 34 before the International Examining Authority.

Upon entry into the national phase, all parts of the international application may be amended under Article 28 or, where applicable, Article 41.

When? Within 2 months from the date of transmittal of the international search report or 16 months from the priority date, whichever time limit expires later. It should be noted, however, that the amendments will be considered as having been received on time if they are received by the International Bureau after the expiration of the applicable time limit but before the completion of the technical preparations for international publication (Rule 46.1).

Where not to file the amendments?

The amendments may only be filed with the International Bureau and not with the receiving Office or the International Searching Authority (Rule 46.2).

Where a demand for international preliminary examination has been/is filed, see below.

How? Either by cancelling one or more entire claims, by adding one or more new claims or by amending the text of one or more of the claims as filed.

A replacement sheet must be submitted for each sheet of the claims which, on account of an amendment or amendments, differs from the sheet originally filed.

All the claims appearing on a replacement sheet must be numbered in Arabic numerals. Where a claim is cancelled, no renumbering of the other claims is required. In all cases where claims are renumbered, they must be renumbered consecutively (Administrative Instructions, Section 205(b)).

The amendments must be made in the language in which the international application is to be published.

What documents must/may accompany the amendments?

Letter (Section 205(b)):

The amendments must be submitted with a letter.

The letter will not be published with the international application and the amended claims. It should not be confused with the "Statement under Article 19(1)" (see below, under "Statement under Article 19(1)").

The letter must be in English or French, at the choice of the applicant. However, if the language of the international application i English, the letter must be in English; if the language of the international application is French, the letter must be in French.

The letter must indicate the differences between the claims as filed and the claims as amended. It must, in particular, indicate, in connection with each claim appearing in the international application (it being understood that identical indications concerning several claims may be grouped),whether

(i) the claim is unchanged;

(ii) the claim is cancelled;

(iii) the claim is new;

(iv) the claim replaces one or more claims as filed;

(v) the claim is the result of the division of a claim as filed.

The following examples illustrate the manner in which amendments must be explained in the accompanying letter:

1. [Where originally there were 48 claims and after amendment of some claims there are 51]:
"Claims 1 to 29, 31, 32, 34, 35, 37 to 48 replaced by amended claims bearing the same numbers; claims 30, 33 and 36 unchanged; new claims 49 to 51 added."

2. [Where originally there were 15 claims and after amendment of all claims there are 11]:
"Claims 1 to 15 replaced by amended claims 1 to 11."

3. [Where originally there were 14 claims and the amendments consist in cancelling some claims and in adding new claims]:
"Claims 1 to 6 and 14 unchanged; claims 7 to 13 cancelled; new claims 15, 16 and 17 added." or
"Claims 7 to 13 cancelled; new claims 15, 16 and 17 added; all other claims unchanged."

4. [Where various kinds of amendments are made]:
"Claims 1-10 unchanged; claims 11 to 13, 18 and 19 cancelled; claims 14, 15 and 16 replaced by amended claim 14; claim 17 subdivided into amended claims 15, 16 and 17; new claims 20 and 21 added."

"Statement under article 19(1)" (Rule 46.4)

The amendments may be accompanied by a statement explaining the amendments and indicating any impact that such amendments might have on the description and the drawings (which cannot be amended under Article 19(1)).

The statement will be published with the international application and the amended claims.

It must be in the language in which the international appplication is to be published.

It must be brief, not exceeding 500 words if in English or if translated into English.

It should not be confused with and does not replace the letter indicating the differences between the claims as filed and as amended. It must be filed on a separate sheet and must be identified as such by a heading, preferably by using the words "Statement under Article 19(1)."

It may not contain any disparaging comments on the international search report or the relevance of citations contained in that report. Reference to citations, relevant to a given claim, contained in the international search report may be made only in connection with an amendment of that claim.

Consequence if a demand for international preliminary examination has already been filed

If, at the time of filing any amendments under Article 19, a demand for international preliminary examination has already been submitted, the applicant must preferably, at the same time of filing the amendments with the International Bureau, also file a copy of such amendments with the International Preliminary Examining Authority (see Rule 62.2(a), first sentence).

Consequence with regard to translation of the international application for entry into the national phase

The applicant's attention is drawn to the fact that, where upon entry into the national phase, a translation of the claims as amended under Article 19 may have to be furnished to the designated/elected Offices, instead of, or in addition to, the translation of the claims as filed.

For further details on the requirements of each designated/elected Office, see Volume II of the PCT Applicant's Guide.

PATENT COOPERATION TREATY

PCT

INTERNATIONAL SEARCH REPORT

(PCT Article 18 and Rules 43 and 44)

Applicant's or agent's file reference	**FOR FURTHER ACTION**	see Notification of Transmittal of International Search Report (Form PCT/ISA/220) as well as, where applicable, item 5 below.
GM/MC/R33-92		
International application No.	International filing date *(day/month/year)*	(Earliest) Priority Date *(day/month/year)*
PCT/SG 00/ 00111	01/08/2000	
Applicant		
INSTITUTE OF MOLECULAR AGROBIOLOGY et al.		

This International Search Report has been prepared by this International Searching Authority and is transmitted to the applicant according to Article 18. A copy is being transmitted to the International Bureau.

This International Search Report consists of a total of 3 sheets.

[X] It is also accompanied by a copy of each prior art document cited in this report.

1. **Basis of the report**

   a. With regard to the **language,** the international search was carried out on the basis of the international application in the language in which it was filed, unless otherwise indicated under this item.

   [ ] the international search was carried out on the basis of a translation of the international application furnished to this Authority (Rule 23.1(b)).

   b. With regard to any **nucleotide and/or amino acid sequence** disclosed in the international application, the international search was carried out on the basis of the sequence listing :

   [X] contained in the international application in written form.

   [ ] filed together with the international application in computer readable form.

   [ ] furnished subsequently to this Authority in written form.

   [X] furnished subsequently to this Authority in computer readble form.

   [X] the statement that the subsequently furnished written sequence listing does not go beyond the disclosure in the international application as filed has been furnished.

   [X] the statement that the information recorded in computer readable form is identical to the written sequence listing has been furnished

2. [ ] **Certain claims were found unsearchable** (See Box I).

3. [ ] **Unity of invention is lacking** (see Box II).

4. With regard to the **title,**

   [X] the text is approved as submitted by the applicant.

   [ ] the text has been established by this Authority to read as follows:

5. With regard to the **abstract,**

   [X] the text is approved as submitted by the applicant.

   [ ] the text has been established, according to Rule 38.2(b), by this Authority as it appears in Box III. The applicant may, within one month from the date of mailing of this international search report, submit comments to this Authority.

6. The figure of the **drawings** to be published with the abstract is Figure No. _____

   [ ] as suggested by the applicant.

   [ ] because the applicant failed to suggest a figure.

   [ ] because this figure better characterizes the invention.

   [X] None of the figures.

ERNATIONAL SEARCH REPORT

Information on patent family members

International Application No
PCT/SG 00/00111

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
US 5792933 A	11-08-1998	NONE	
WO 9639021 A	12-12-1996	AU 700848 B	14-01-1999
		AU 6100096 A	24-12-1996
		EP 0777412 A	11-06-1997
		US 6096950 A	01-08-2000

International Application No
PCT/SG 00/00111

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication,where appropriate, of the relevant passages	Relevant to claim No.
A	DANG, P.M. ET AL.: "Expression of a "cotton fiber specific" gene, Gh-1, in transgenic tobacco and cotton" PLANT PHYSIOLOGY, vol. 111, 1996, page 55 XP000974214 the whole document	1-3
A	JOHN, M.E. AND CROW, L.J.: "Gene expression in cotton (Gossypium hirsutum L.) fiber: Cloning of the mRNAs" PROC.NATL.ACAD.SCI.USA, vol. 89, July 1992 (1992-07), pages 5769-5773, XP002098262 the whole document	1-3
A	KOGA-BAN, Y. ET AL.: "cDNA sequences of three kinds of ß-tubulins from rice" DNA RESEARCH, vol. 2, no. 1, 1995, pages 21-26, XP000907121 the whole document	1-3

INTERNATIONAL SEARCH REPORT

International Application No
PCT/SG 00/00111

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 C12N15/11

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 7 C12N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)
EPO-Internal, WPI Data, PAJ, BIOSIS, EMBASE, MEDLINE, SCISEARCH

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	US 5 792 933 A (MISSISSIPPI STATE UNIVERSITY) 11 August 1998 (1998-08-11)	1
A	column 2, line 12 -column 6, line 15	2,3
Y	WO 96 39021 A (THE MONSANTO COMPANY) 12 December 1996 (1996-12-12)	1
A	page 8, line 1 -page 13, line 28	2,3
A	RINEHART, J.A. ET AL.: "Tissue-specific and developmental regulation of cotton gene FbL2A" PLANT PHYSIOLOGY, vol. 112, no. 3, November 1996 (1996-11), pages 1331-1341, XP000974218 the whole document	1-3

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[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

° Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance
"E" earlier document but published on or after the international filing date
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
"O" document referring to an oral disclosure, use, exhibition or other means
"P" document published prior to the international filing date but later than the priority date claimed
"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.
"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
19 February 2001	14/03/2001

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL – 2280 HV Rijswijk Tel. (+31–70) 340–2040, Tx. 31 651 epo nl, Fax: (+31–70) 340–3016	Donath, C

Form PCT/ISA/210 (second sheet) (July 1992)

(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization
International Bureau

(43) International Publication Date
7 February 2002 (07.02.2002)

PCT

(10) International Publication Number
WO 02/10377 A1

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(21) International Application Number: PCT/SG00/00111

(22) International Filing Date: 1 August 2000 (01.08.2000)

(25) Filing Language: English

(26) Publication Language: English

(71) Applicant *(for all designated States except US)*: **INSTITUTE OF MOLECULAR AGROBIOLOGY** [SG/SG]; 1 Research Link, National University of Singapore, Singapore 117604 (SG).

(72) Inventors; and

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(74) Agent: **MIRANDAH, Gladys**; Ella Cheong & G. Mirandah, 111 North Bridge Road, #22-01/02/03 Peninsula Plaza, Singapore 179098 (SG).

(81) Designated States *(national)*: AE, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CR, CU, CZ, DE, DK, DM, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, TZ, UA, UG, US, UZ, VN, YU, ZA, ZW.

(84) Designated States *(regional)*: ARIPO patent (GH, GM, KE, LS, MW, MZ, SD, SL, SZ, TZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG).

Published:
— *with international search report*

For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.

WO 02/10377 A1

(54) Title: ISOLATION AND CHARACTERIZATION OF A FIBER-SPECIFIC β-TUBULIN PROMOTER FROM COTTON

(57) Abstract: The present invention relates to a cotton β-tubulin gene CFTUB2, and active fragments thereof. These promoters show strong fiber-specific activity.

ISOLATION AND CHARACTERIZATION OF A FIBER-SPECIFIC β-TUBULIN PROMOTER FROM COTTON

Technical Field

The present invention relates to the field of plant molecular biology, in particular to transgenic plants and promoters useful in creating transgenic plants, and more particularly to fiber-specific promoters.

Background of the Invention

Cotton is the most extensively used natural fiber in the textile industry. Annual production of cotton worldwide is over 100 million bales valued at 45 billion U.S. dollars. Although significant improvements have been made in quality and yield of the fibers by means of classical breeding in the past decades, the potential for further improving fiber properties through classical breeding is limited due to requirements for species compatibility and available traits. Genetic engineering provides novel approaches for further improving cotton by introducing genes to create new germplasms with highly desirable characteristics.

Cotton fibers (seed hairs) are single-cell trichomes that undergo rapid and synchronous elongation. Cortical microtubules provide spatial information necessary for the alignment of cellulose microfibrils that confine and regulate cell elongation [Giddings and Staehelin, 1991; Cyr and Palevitz, 1995; Fisher and Cyr, 1995]. Fiber development consists of four overlapping stages (i.e. initiation, primary cell wall formation, secondary

cell wall formation and maturation) [Basra and Malik, 1984]. Tubulins and actins may play functionally important roles in developing fiber cells. Mature fiber is a biological composite of cellulose, water, small quantities of proteins, pectins, hemicellulose, mineral substances, wax, small amounts of organic acids, sugars, and pigments that provides excellent wearability and aesthetics [Arthur, 1990; Basra and Malik, 1984; Ryser, 1985]. Many genes are required for the fiber differentiation and development. These genes are differentially expressed during different stages of the fiber development, and so far only a few of the genes involved in the biosynthesis of the large numbers of fiber-specific structural proteins, enzymes, polysaccharides, waxes or lignins have been identified [John and Crow, 1992; John, 1996a; Song and Allen, 1997; Ma et al., 1997; Kawai et al., 1998; Whittaker and Triplett, 1999]. These isolated genes may be considered as having potential application in cotton fiber improvement due to the character of their fiber-specific expression. For example, John has been using fiber-specific gene promoters to produce genetically engineering cotton for altered fibers [John, 1996b, 1997a, 1997b].

A promoter is a DNA fragment that determines temporal and spatial specificity of gene expression during plant and animal development. Many tissue-specific genes and their promoters were identified and isolated from a wide variety of plants and animals over the past decade, including some cotton tissue-specific genes and promoters (Loguerico et al., 1999; Kawai et al., 1998; Song and Allen, 1997; Ma et al, 1997; John, 1996a; Rinehart et al., 1996; Hasenfratz et al, 1995; John and Peterson, 1994; John and Crow, 1992). A few promoters have been shown to control gene expression in a fiber-specific manner in cotton (Rinehart et al., 1996; John, 1996a; John and Crow, 1992). Some plant tissue-specific promoters can be utilized to

express foreign proteins in specific tissues in a developmentally regulated pattern [John, 1996b, 1997a, 1997b].

Summary of the Invention

A fiber-specific gene (named CFTUB2), encoding β-tubulin, was isolated from cotton. The isolated complete CFTUB2 cDNA is 1.623 kb in length including 1.338 kb of open reading frame. Based on the CFTUB2 cDNA sequence, two CFTUB2 promoter fragments (1.433 kb and 0.984 kb) were isolated from cotton. The two CFTUB2 promoter fragments (1.3 and 0.9 kb) were fused with the GUS gene to construct gene expression vectors for analyzing the function of the promoter. Transgenic cotton and tobacco plants with the CFTUB2 promoter/GUS fusion genes were identified by Southern blot hybridization. In all the transgenic cotton plants studied, GUS activity was detected only in young fibers, but not in the flower organs such as anthers, petals and sepals, or in leaves and roots. This result, together with Northern blot analysis, indicates that the CFTUB2 promoter is fiber-specific in cotton. The promoter controls specific gene expression at the transcriptional level in cotton fibers. The isolated promoter may be used in improving cotton fibers to create new cotton varieties with high fiber quality and yield by gene manipulation.

Brief Description of the Figures

Figure 1 shows the nucleotide sequence of the cotton CFTUB2 gene cDNA (1623 bp; SEQ ID NO: 1).

Figure 2 shows the nucleotide sequence of the isolated 1433 bp CFTUB2 promoter fragment (SEQ ID NO: 2. The 984 bp fragment corresponds to nucleotides 449-1433 of this sequence.

Figure 3 shows constructs of the CFTUB2 promoter fused with the gus gene in expression vectors.

Detailed Description

The CFTUB2 promoter is an active fiber-specific promoter in cotton. Results of a Northern blot analysis of cDNAs from a variety of cotton tissues showed that a cDNA clone comprising the CFTUB2 gene was strongly expressed in young fibers of 8 and 14 days postanthesis (DPA), and also expressed in young ovules of 4, 8 and 14 DPA, but less or not at all in other tissues. Sequencing of the cDNA clone revealed that it was 1623 bp in length containing a open reading frame of 1338 bp (Figure 1). Comparing the nucleotide and predicted polypeptide sequences of the cotton CFTUB2 with the data banks, it was found that the CFTUB2 cDNA shared 96%-98% homology at the amino acid level and over 78% homology at the nucleotide level with the known β-tubulin cDNAs and genes from other plants (such as Arabidopsis, tobacco, rice, soybean, maize, potato, carrot, etc.) [Liaud et al., 1992; Snustad et al., 1992; Villemur et al, 1994; Tonoike, et al., 1994; Taylor et al., 1994; Kang et al., 1994; Okamura et al., 1997; Chu et al., 1998; Okamura et al., 1999].

The transcripts of the CFTUB2 gene exhibited the highest accumulation in cotton young fibers of 8 DPA, and then there was a visible decrease in the accumulation of the gene products (mRNA) with further development of the fibers. Comparison of gene expression in different developmental stages of cotton ovules also showed that the gene transcripts accumulated more in 8 DPA ovules than in 4 and 14 DPA, and there was a gradual and visible decrease to an undetectable level in the accumulation of gene products with fiber development from 8 DPA to 28 DPA. This suggests that the gene is specifically expressed with a strict regulation at the transcriptional level during cotton fiber and ovule development, as with other cotton fiber-specific genes [Whittaker and Triplett, 1999; Shin and Brown, 1999; Kawai et

al., 1998; John, 1996a; Song and Allen, 1997; Ma et al, 1997; Rinehart et al., 1996; John and Crow, 1992].

Two fragments in the promoter region were isolated and cloned into pGEM-T vector, respectively. One fragment of the CFTUB2 promoter was 1433 bp in length (Figure 2), and another was 984 bp long. Both fragments functioned as active, fiber-specific promoters. The constructs of CFTUB2 promoter/GUS fusion gene were used to transform tobacco and cotton by Agrobacterium-mediated gene transfer, using the pBI121 vector containing CaMV35S promoter/GUS fusion as a positive control. Consistent with the results from Northern blot analysis, the GUS gene driven by CFTUB2 promoter specifically expressed in the young fibers, but not in other tissues, in all the 31 transgenic cotton plants studied, while the GUS activity was detected in all the tissues of positive control cotton plants (35S:GUS). A total of 36 transformed cotton plants were obtained and transplanted in soil to grow to maturation. Similarly, it was found that under the CFTUB2 promoter, GUS gene activity was only detected in the seeds in all of the 15 transgenic tobacco plants studied, suggesting the CFTUB2 promoter activity was also tissue-specific in tobacco (the cotton fiber, being an elongated hair of the seed coat, finds histological correspondence in the tobacco seed coat). This result, together with the above Northern blot analysis, indicates that the CFTUB2 promoter controls gene specific expression at the transcriptional level in cotton fibers.

Accordingly, one embodiment of the present invention is a fiber-specific promoter obtained from the cotton fiber β-tubulin gene CFTUB2.

Another embodiment of the present invention is a fiber-specific promoter comprising a 1433 kb active fragment of the cotton fiber CFTUB2 gene promoter.

Another embodiment of the present invention is a fiber-specific promoter comprising a 984 kb active fragment of the cotton fiber CFTUB2 gene promoter.

Stll another embodiment of the present invention is a promoter that is cotton fiber-specific comprising an active fragment of the CFTUB2 promoter fragment of SEQ ID NO: 2. An active fragment is a sequence of shorter length than SEQ ID NO: 2 which still retains activity as a fiber-specific promoter in cotton. A fragment can comprise excisions, deletions, truncations or substitutions of the sequence of SEQ ID NO: 2, or a combination of these. A preferred active fragment is the fragment consisting of nucleotides 449-1433 of SEQ ID NO: 2.

The promoters of the present invention are useful in creating transgenic cotton having altered fiber characteristics. The use of the fiber-specific promoters of the present invention permits selective expression of a transgene in the cotton fiber, permitting greater latitude in the types of transgenes employed. Selective expression avoids problems such as the metabolic burden imposed on a transgenic plant by systemic expression of a transgene, or the adverse effects of the expression of a transgene in non-fiber tissues. Examples for expressing desirable genes in cotton fiber, but not in other parts of the cotton plants include: (1) anthocynin genes for colored cotton, (2) silk protein genes from silk worm or spiders for increased strength of cotton fiber, (3) and biosynthesis of polyhydroxybutrate in cotton fiber for improved thermal properties and insulating characteristics [John, et al., 1996]. There are numerous examples in the art of fiber-enhancing genes that could be advantageously linked to the promoters of the present invention, and used to transform cotton using well-known techniques (see, e.g., Umbeck, 1992), to achieve expression of

the transgene in transgenic cotton fibers. See e.g., John, 1996b, 1997a, 1997b; John et al., 1996.

EXAMPLE 1: Isolation of fiber-specific cDNA encoding CFTUB2 sequences expressed early during fiber development of cotton

Cotton seeds were surface-sterilized with 70% ethanol for 30-60 seconds and 10% H_2O_2 for 30-60 minutes, followed by washing with sterile water. The seeds germinated on ½ MS medium on light at 28°C in a culture room, and cotyledons and hypocotyls cut from sterile seedlings were used as transformation explant materials. Cotton plants were grown in pots for DNA and RNA extraction.

Total RNA was extracted from young fibers, ovaries, anthers, petals, sepals, leaves and roots of cotton by using the guanidinium thiocyanate method or SV Total RNA Isolation System (Promega). Poly(A)+RNA was purified by using oligo(dT)-cellulose spin columns from an mRNA purification kit (Pharmacia Biotech). Cotton cDNA was synthesized by using a cDNA synthesis kit (Pharmacia Biotech). Cotton cDNA libraries were constructed by inserting the cDNA fragments into the ZAP express vector (Stratagene).

Poly(A)+ RNAs from cotton young fibers of about 8 and 14 days postanthesis (DPA), respectively, were converted to cDNAs which were used to construct cotton cDNA libraries. From the fiber cDNA libraries, about 200 cDNA clones were randomly picked out and subsequently sequenced. Some clones with potential involvement in cell expansion were selected according to the sequence data.

To find cDNA clones whose transcripts are specifically expressed in cotton fibers, the expression pattern of the

selected cDNA clones was analyzed by Northern blot hybridization with total RNAs isolated from cotton fibers, ovules, anthers, petals, sepals, squares, leaves and roots, using probes from the clones. RNA samples from the different cotton tissues were separated on agarose-formaldehyde gels, and transferred onto Hybond-N nylon membranes by capillary blotting. RNA Northern blots were hybridized in ExpressHyb solution (Clontech) at 68°C with ^{32}P cDNA probes prepared by random labeling (Promega Prime-a-Gene Labeling System). After hybridization, the blots were washed at 68°C in 0.1 x SSC, 0.5% SDS for 30-60 minutes. The experimental results showed that one cDNA clone strongly expressed in young fibers of 8 and 14 DPA, and also expressed in young ovules of 4, 8 and 14 DPA, but less or not at all in other tissues.

PCR fragments and cDNA fragments were subcloned into vectors, and plasmid DNA prepared with a Qiagen plasmid kit was used as templates in PCR reactions. The PCR products were sequenced by autosequencer. Sequencing of the cDNA clone revealed that it was 1623 bp in length containing a open reading frame of 1338 bp, and identical to the β-tubulin gene (Figure 1). This is the first CFTUB2 cDNA clone isolated from cotton. Comparing the nucleotide and predicted polypeptide sequences of the cotton CFTUB2 with the data banks, it was found that the CFTUB2 cDNA shared 96%-98% homology at the amino acid level and over 78% homology at the nucleotide level with the known β-tubulin cDNAs and genes from other plants (such as Arabidopsis, tobacco, rice, soybean, maize, potato, carrot, etc.) [Liaud et al., 1992; Snustad et al., 1992; Villemur et al, 1994; Tonoike, et al., 1994; Taylor et al., 1994; Kang et al., 1994; Okamura et al., 1997; Chu et al., 1998; Okamura et al., 1999].

Total RNAs from different tissues of cotton were used to reverse-transcribe first-strand cDNAs which were used as templates in differential display PCR reactions. Differential display analysis was carried out by using a differential display kit (Clontech). First-strand cDNA was synthesized with 2 pg total RNA as starting materials of reverse transcription and oligo(dT) as primers at 42°C for 1 hour. Differential display PCR reactions were carried out with a initial cycle consisting of 94°C for 5 minutes, 40°C for 5 minutes and 68°C for 5 minutes, followed by two cycles consisting of 94°C for 2 minutes and 40°C for 5 minutes and 68°C for 5 minutes, and then 25 cycles consisting of 94°C for 1 minute and 60°C for 1 minute and 68°C for 2 minutes, and a final extension at 68°C for 7 minutes. Target differential display bands were excised and reamplified for further analysis. Reproducible fiber-specific differential display products were targeted for further analysis. The cDNA in each target band was harvested and regenerated by PCR amplification. The isolated cDNA was subsequently subcloned into vectors and sequenced.

The Northern blot analysis showed that the transcripts of the CFTUB2 gene exhibited a highest accumulation in cotton young fibers of 8 DPA, and then there was a visible decrease in the accumulation of the gene products (mRNA) with further development of the fibers. Comparison of gene expression in different developmental stages of cotton ovules also showed that the gene transcripts accumulated more in 8 DPA ovules than in 4 and 14 DPA, and there was a gradual and visible decrease to an undetectable level in the accumulation of gene products with fiber development from 8 DPA to 28 DPA. This suggests that the gene is specifically expressed with a strict regulation at the transcriptional level during cotton fiber and ovule development, as seen with other cotton fiber-specific genes [Whittaker and

Triplett, 1999; Shin and Brown, 1999; Kawai et al., 1998; John, 1996a; Song and Allen, 1997; Ma et al, 1997; Rinehart et al., 1996; John and Crow, 1992].

Example 2: Isolation of the CFTUB2 promoter

Based on the screened CFTUB2 cDNA sequence, the CFTUB2 promoter was isolated from cotton Genome Walker libraries by Genome Walker PCR.

Total DNA was extracted and purified from leaves of cotton plants by using the following method. Liquid N_2 was added to 4 g of leaf tissues, and the leaves were homogenized thoroughly. 20 ml ice-cold extraction buffer (63 g/L glucose, 0.1 M Tris.HCl (pH 8.0), 5 mM EDTA, 20 g/L PVP-40, 1 g/L DIECA, 1 g/L ascorbic acid, 2ml/L, betamercaptoethanol) was added to the homogenized tissues in a 50 ml tube and centrifuged at 2500 rpm for 15 minutes. After removing the supernatant, 10 ml lysis buffer was added to each tube. The resuspended pellets were incubated at 65°C for 30 minutes. 10 ml chloroform was added to each tube, mixed with the samples and centrifuged at 3500 rpm for 10 minutes. The supernatant was transferred to a clean tube, and chloroform extraction was repeated one more time. The supernatant was transferred to a clean tube, and 0.6 volume isopropanol was added to each tube for DNA precipitation. After centrifuging at 3500 rpm for 30 minutes, the DNA was washed with 70% ethanol. The isolated genomic DNA was then dissolved in sterile water or TE (10 mM Tris.HCl, 1 mM EDTA) for use.

Cotton genomic DNA libraries were constructed from leaves of cotton plants. DNA was partially digested with BamH I, and the DNA fragments were cloned in the *BamH* I site of the ZAP expression vector (Stratagene).

Genome Walker libraries were constructed by using Universal Genome Walker kit (Clontech). Genomic DNA from leaves

of cotton plants was digested with five restriction enzymes respectively, and then purified by phenol/chloroform and precipitated by ethanol. Digested DNA was ligated to Genome Walker adaptors. Two rounds of Genome Walker PCR reactions were carried out successively. 1 µl of each Genome Walker DNA library was used as templates in the primary PCR, and the primary PCR products were used as templates in secondary PCR. The PCR was started at 95°C for 1 minute, followed by 35 cycles consisting of 95°C for 15 seconds and 68°C for 4 minutes, and a final extension at 68°C for 6 minutes. Target PCR bands were cut out and purified by Geneclean kit (Bio 101).

Two fragments in the promoter region were isolated and cloned into pGEM-T vector, respectively. One fragment of the CFTUB2 promoter was 1433 bp in length Figure 2, and another was 984 bp long. Figure 2. A Hind III site and a BamH I site were created at the 5'-end and 3'-end of the 0.9 kb CFTUB2 promoter fragment of cotton respectively by PCR method. The Hind III/BamH I fragment was initially subcloned into pGEM-T vector (Promega). Plasmid DNA containing the CFTUB2 promoter fragment was digested with Hind III and BamH I, and the digested fragment was isolated by agarose gel electrophoresis. A chimeric CFTUB2 promoter/GUS construct was generated by insertion of the fragment, replacing CaMV 35S promoter, into the Hind III/BamH I sites of pBI121 vector.

The 1.3 kb of BamH I/BamH I CFTUB2 promoter fragment was initially subcloned into the pGEM-T vector (Promega). Plasmid DNA containing the CFTUB2 promoter fragment was digested with BamH I, and the digested fragment was isolated by agarose gel electrophoresis. A chimeric CFTUB2 promoter/GUS construct was generated by insertion of the fragment into the BamH I site of pBI101 vector.

Example 3: Functional analysis of the CFTUB2 promoter

In order to characterize the function of CFTUB2 promoter in fiber-specific expression of the CFTUB2 gene, a 1.3 kb of fragment and a 0.9 kb of fragment of the CFTUB2 promoter were fused with gus coding sequence in the gene expression vector pBI101 or pBI121 (deleting CaMV35S promoter), respectively (Figure 3). The constructs of CFTUB2 promoter/GUS fusion gene were used to transform tobacco and cotton by Agrobacterium-mediated gene transfer, using the pBI121 vector containing CaMV35S promoter/GUS fusion as a positive control. The CaMV35S promoter is active in all the tissues of cotton and other plants and is a constitutive promoter [Odell et al., 1985; Ow et al., 1987; McCabe and Martinell, 1993]. A binary vector containing either a CFTUB2 promoter/GUS fusion gene or the CaMV35S promoter/GUS control control was transferred into Agrobacterium tumefaciens strain LBA 4404. Cotton explants for transformation were obtained from cotton seedlings grown as in Example 1. Tobacco explant material was obtained from tobacco seedlings. Tobacco seeds were surface-sterilized with 70% ethanol for 30-60 seconds and 0.1% $HgCl_2$ for 15 minutes, followed by washing with sterile water. The seeds germinated on ½ MS medium on light at 28°C in culture room, and leaves cut from sterile seedlings for use as explants for transformation. Cotton cotyledon and hypocotyl explants and tobacco leaf explants were transformed by the Agrobacterium with the vectors, and transformed plants were transplanted to soil in greenhouse for growing to maturity.

Tobacco leaves were cut into about 2x2 cm pieces, and immersed in Agrobacterium suspension for 5 minutes. The infected tobacco explants were cultivated on MS medium with 1 mg/L 6-BA for 48 hours at 28°C, and then transferred onto selection MS medium containing 100 mg/L kanamycin and 1 mg/L 6-

BA for 20-30 days for selecting transformed shoots (kanamycin-resistant shoots). The transformed shoots were cut from the calli and rooted on MS medium with 50-100 mg/L kanamycin. The transformed tobacco plants were transplanted to soil in greenhouse for growing to maturity.

The cotyledon and hypocotyl were used as explants for cotton transformation. Cotton seeds were surface-sterilized with 70% ethanol for 30 seconds and 10% H_2O_2 for 60 minutes, followed by washing with steril water. These seeds were incubated in the sterile water at 28°C. After over night, the seeds sprouted. The embryos were taken out and put on the IM medium (1/2 {MS (macronutriants, micronutrients, EDTA-Fe) + VB1 10mg/L + VB6 1mg/L + VPP 1mg/L + Myo-Insitol 100mg/L} + phytogel 2g/L pH = 6.4) at 28°C for 7 days. The cotyledon and hypocotyl of cotton were used as explants for transformation. After cutting into 5 mm^2 (mm) piece, the explants were soaked in the Agrobacterium tumefaciens strain LBA 4404 suspension (OD_{600} = 0.2 - 0.4) for 15 minutes. Then the explants were put on CM medium (MS (macronutriants, micronutrients, EDTA-Fe) + VB1 10mg/L + VB6 1mg/L + VPP 1mg/L + Myo-Instiol 100mg/L + 2.4-D 0.1mg/L + KT 0.1mg/L + Glucose 30g/L + $MgCl_2$ 0.7mg/L + phytogel 2g/L pH = 6.4) at 24°C for 2 days. After washing with liquid MS medium, the explants were put on the SM medium (MS (macronutriants, micronutrients, EDTA-Fe) + VB1 10mg/L + VB6 1mg/L = VPP 1mg/L + Myo-Insitol 100mg/L + 2.4-D 0.1mg/L + KT 0.1mg/L + Glucose 30g/L + $MgCl_2$ 0.7mg/L + phytogel 2g/L + Kanamycin 50mg/L + Cefutoxime 200mg/L pH = 6.4) on light at 28°C in culture room for selecting and the subculture was per month. After 2-3 months subculturing on SM, the calli were induced from explants. The calli were transferred on DM medium (MS (macronutriants, micronutrients, EDTA-Fe) + VB1 10mg/L + VB6 1mg/L + VPP 1mg/L + Myo-Insitol 100mg/L + KNO_3 19g/L + $MgCl_2$

0.7mg/L + Glucose 30g/L + phytogel 3g/L pH = 6.4) and subcultured per month. After about 5 months, the somatic embryos begin to form. Continuing to culture the young embryos on DM medium until they develop into maturity. The mature embryos were transferred on GM medium (1/2 {MS (macronutriants, micronutrients, EDTA-Fe) + VB1 10mg/L + VB6 1mg/L + VPP 1mg/L + Myo-Insitol 100mg/L) + NAA 0.01mg/L + Glucose 30g/L + phytogel 3.5g/L pH = 6.4) in the box for developing into plantlets. And then the plantlets were transplanted in the soil for the plant growing and collecting the transgenic seeds.

Transgenic tobacco and cotton plants possessing the chimeric CFTUB2 promoter/GUS gene (or 35S:GUS gene), and non-transformed plants as negative controls, were analyzed by DNA Southern blot hybridization and by GUS histochemical assay. Total genomic DNA from cotton and tobacco leaves were digested with restriction enzymes, separated on agarose gels, and transferred onto Hybond-N nylon membranes by capillary blotting. DNA Southern blots were hybridized in ExpressHyb solution (Clontech) at 68°C with ^{32}P-DNA probes prepared by random labeling (Promega Prime-a-Gene Labeling System). After hybridization, the blots were washed at 68°C in 0.1 x SSC, 0.5% SDS for 30-60 minutes. The ^{32}P-labeled nylon membranes were exposed to X-ray film at - 70°C for autoradiography. The results of Southern blot analysis demonstrated that CFTUB2 promoter/GUS gene was integrated into tobacco and cotton genomes. Total of 325 transformed cotton plants, which belong to 31 transformed lines, were obtained and transplanted in soil to grow to maturation.

Histochemical assays for GUS activity in transgenic tobacco and cotton plants were conducted according to the protocol described previously by Jefferson et al. (1987) with some modifications. Fresh tissues from the plants were incubated

in X-gluc (5-bromo-4-chloro-3-indolylglucuronide) solution consisting of 0.1 M sodium phosphate (pH 7.0), 10 mM ethylene diaminetetraacetic acid (EDTA), 0.5 mM potassium ferrocyanide and 0.5 mM potassium ferricyanide, and 0.1 % X-gluc (Clontech chemical) overnight. The stained plant materials were then cleared and fixed by rinsing with 100% and 70% ethanol successively, and the samples were examined and photographed directly or under a microscope. Consistent with the results from Northern blot analysis, the GUS gene driven by CFTUB2 promoter specifically expressed in the young fibers, but not in other tissues, in all the 31 transgenic cotton plants studied, while the GUS activity was detected in all the tissues of positive control cotton plants (35S:GUS). A total of 36 transformed cotton plants were obtained and transplanted in soil to grow to maturity, all of which had detectable GUS activity only in the young fibers, not in the flower organs such as anthers, petals and sepals, or in leaves and roots. Similarly, it was found that under the CFTUB2 promoter, GUS gene activity was only detected in the seeds in all of the 15 transgenic tobacco plants studied, suggesting the CFTUB2 promoter activity was also tissue-specific in tobacco. This result, together with the above Northern blot analysis, indicates that the CFTUB2 promoter controls gene specific expression at the transcriptional level in cotton fibers.

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We claim:

1. A promoter that is cotton fiber-specific, comprising the promoter of the cotton β-tubilin gene CFTUB2.

2. A promoter that is cotton fiber-specific, comprising a 1433 kb fragment of the promoter of the cotton β-tubilin gene CFTUB2 having the sequence of SEQ ID NO: 2.

3. A promoter that is cotton fiber-specific, comprising a 984 kb fragment of the promoter of the cotton β-tubilin gene CFTUB2 having the sequence of nucleotides 449-1433 of SEQ ID NO: 2.

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Fig. 1

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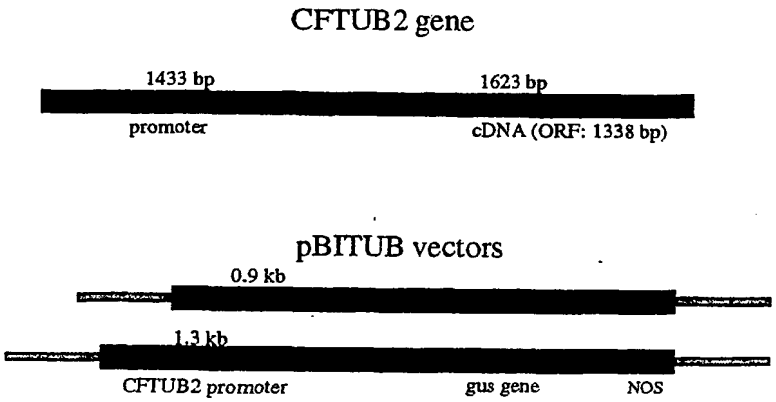
Fig. 2


CFTUB2 gene
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1623 bp
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1.3 kb
CFTUB2 promoter
gus gene
NOS

Fig. 3

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Chen, N. H.
Liu, J. W.

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INTERNATIONAL SEARCH REPORT

International Application No
PCT/SG 00/00111

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 C12N15/11

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 7 C12N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)
EPO-Internal, WPI Data, PAJ, BIOSIS, EMBASE, MEDLINE, SCISEARCH

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	US 5 792 933 A (MISSISSIPPI STATE UNIVERSITY) 11 August 1998 (1998-08-11)	1
A	column 2, line 12 -column 6, line 15	2,3
Y	WO 96 39021 A (THE MONSANTO COMPANY) 12 December 1996 (1996-12-12)	1
A	page 8, line 1 -page 13, line 28	2,3
A	RINEHART, J.A. ET AL.: "Tissue-specific and developmental regulation of cotton gene FbL2A" PLANT PHYSIOLOGY, vol. 112, no. 3, November 1996 (1996-11), pages 1331-1341, XP000974218 the whole document	1-3

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[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

° Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

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"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
19 February 2001	14/03/2001

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL – 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Donath, C

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C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication,where appropriate, of the relevant passages	Relevant to claim No.
A	DANG, P.M. ET AL.: "Expression of a "cotton fiber specific" gene, Gh-1, in transgenic tobacco and cotton" PLANT PHYSIOLOGY, vol. 111, 1996, page 55 XP000974214 the whole document	1-3
A	JOHN, M.E. AND CROW, L.J.: "Gene expression in cotton (Gossypium hirsutum L.) fiber: Cloning of the mRNAs" PROC.NATL.ACAD.SCI.USA, vol. 89, July 1992 (1992-07), pages 5769-5773, XP002098262 the whole document	1-3
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WO 9639021 A	12-12-1996	AU 700848 B	14-01-1999
		AU 6100096 A	24-12-1996
		EP 0777412 A	11-06-1997
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